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MORPHOLOGY, GENETICS, AND MALE MATING SUCCESS COMPARED BETWEEN *ANTHOCORIS MUSCULUS* AND *A. ANTEVOLENS* (HEMIPTERA: HETEROPTERA: ANTHOCORIDAE)

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Abstract.—The predatory true bugs *Anthocoris antevolens* White and *A. musculus* (Say) (Hemiptera: Heteroptera: Anthocoridae) are geographically widespread species in North America having broadly overlapping ranges. The two species are similar in coloration, size, host-plant use, and general appearance of the male genitalia. They are separated in keys by characteristics of the pubescence on the hemelytra: *A. antevolens*, pubescence long and dense; *A. musculus*, pubescence short and sparse. However, the extensive variability in this trait, in combination with similarities in other traits, has led to questions about whether *A. antevolens* and *A. musculus* are actually distinct species. We compared behavioral, morphological, and molecular genetic traits among specimens collected from four geographic regions, whose appearance would identify them as *A. musculus* (from three populations: Maine, Michigan, Montana) or as *A. antevolens* (from one population: central Washington). We included for comparison results for three populations of *A. antevolens* shown in earlier publications to differ in behavior, morphology, and mitochondrial DNA. Our results showed that identifications made using pubescence traits often failed to parallel variation in other characteristics, notably appearance of the male genitalia, mating success, and DNA sequences. In sum, our results indicate that variation among populations of *A. antevolens* in morphological, behavioral, and genetic traits may often exceed differences in those same traits between *A. musculus* and *A. antevolens*, if identifications are made using available keys.

Key Words: Insecta, morphometrics, genitalia, reproductive isolation, geographic variation, mtDNA, cryptic species

Henry (1988) listed 12 species of *Anthocoris* Fallén (Hemiptera: Heteroptera: Anthocoridae) in the continental United States and Canada. The two most geographically widespread species are *A. antevolens* White and *A. musculus* (Say). Literature records indicate that *A. antevolens* is found throughout Canada, south into the New England states in the east, north into Alaska, and south into Arizona and California in the west (Kelton 1978, Henry 1988, Lewis et al. 2005). *Anthocoris musculus* is also transcontinental in Canada (Kelton 1978). In the United States, *A. musculus* has been recorded from Alaska, the New England states, and the northern midwest states (Kelton 1978, Henry 1988, Lewis et al. 2005). *Anthocoris antevolens* is more common in western than in eastern

North America, in contrast to *A. musculus*, which is more common in the east (Hill 1957, Kelton 1978).

The taxonomic status of *A. antevolens* and *A. musculus* is unclear. *Anthocoris musculus* was described by Say (1832) from insects inhabiting the Great Lakes Region. *Anthocoris antevolens* was described by White (1879) from insects collected in California; White made no mention of *A. musculus* in his description. The two species are very similar in size and shape of the male genitalia (Hill 1957, Kelton 1978, T.M.L. unpublished data) and in shape of the female's copulatory tube (Ke and Bu 2007). Male and female genitalia differ strikingly in appearance from genitalia in virtually all other North American species of *Anthocoris* (Hill 1957, Kelton 1978, T.M.L. unpublished data). *Anthocoris antevolens* and *A. musculus* are separated in keys by length and density of pubescence on the hemelytra (Hill 1957, Kelton 1978): pubescence in *A. antevolens* is long and dense, whereas that in *A. musculus* is short and sparse. However, this trait is highly variable among populations within this complex, and "intermediate" forms have been collected (Harper 1959). Anderson (1962) and Hill (1957) both questioned whether *A. musculus* and *A. antevolens* are separate species or geographic forms of one species. The confusion has recently become even more pronounced. We now know that *A. antevolens* itself is a complex of reproductively isolated species, each of whose appearance would identify the species as *A. antevolens* in available keys (Horton and Lewis 2005; Horton et al. 2005, 2007). The reproductive isolation occurs even between sympatric populations thought originally to comprise a single species, *A. antevolens* (Horton and Lewis 2005, Horton et al. 2005).

The present study is part of a larger project having objectives to examine taxonomic questions within the *A. antevolens – A. musculus* species complex. Here, we compare mating success, morphological traits, and DNA sequence data among populations of *A. musculus* from Maine, Michigan, and Montana, with those same traits in specimens from a population in Washington that keys readily to *A. antevolens*. We conducted mating trials to determine whether males from one population could inseminate females from other populations. We have shown complete pre-insemination isolation between populations of *A. antevolens* using these methods (Horton and Lewis 2005, Horton et al. 2005). We also compared body size and length of the phallus for specimens from each population; the latter measure differs significantly among populations of *A. antevolens* (Horton and Lewis 2005, Horton et al. 2007). We collected mitochondrial DNA (mtDNA) sequences from specimens in each population to assess the extent of divergence among populations. We previously used mtDNA to confirm genetic divergence of *A. antevolens* populations that also diverge in behavioral and morphological traits (Horton et al. 2007). In all of our assessments, we hypothesized that the trait being measured would differ less among the three *A. musculus* populations than between the *A. musculus* populations and the *A. antevolens* population.

Finally, we include for comparison results published elsewhere (Horton et al. 2005, 2007) for three reproductively isolated populations of *A. antevolens* that vary in morphology and molecular genetics. These data allow us to show that variation in traits among populations identified as *A. antevolens* may often exceed differences in those same traits between *A. musculus* and certain populations of *A. antevolens*.

Materials and Methods

Source of insects and rearing.—Insects from four populations were collected from *Salix* (Salicaceae) in the summer of 2003, with a few additional specimens for the mtDNA studies taken from pinned specimens available in the collection of T.M.L. (see below). Specimens from three populations (hereafter, Maine, Michigan, Montana) keyed readily to *A. musculus* in Hill (1957) and Kelton (1978), based upon characteristics of pubescence on the hemelytra; the fourth population (Washington) keyed to *A. antevolens*. Field-collected insects were used to establish laboratory cultures of each population. We collected 16 specimens of *A. musculus* from Searsport, Waldo County, ME, of which 8 females deposited eggs in the laboratory. We had considerably more difficulty finding *A. musculus* at the other two sites. The Michigan insects (4 females, of which 3 oviposited) were collected from Beal City, Isabella County, MI. The Montana insects (11 specimens, of which 5 were egg-laying females) were collected from Lolo Creek, 22 km west of Lolo, Missoula County, MT.

The Washington population (*A. antevolens*) was collected from Union Gap, Yakima County, WA, and included 10 egg-laying females. This population of *A. antevolens* has been used in behavioral and morphological studies published elsewhere (designated as the UG or Union Gap population in Horton et al. 2005, 2007). The population was chosen specifically because specimens key readily to *A. antevolens* based upon pubescence, and because preliminary inspection showed phallus lengths in males to be similar to lengths in males of *A. musculus* from the three study populations. Phallus length is quite variable among populations of *A. antevolens* (Horton and Lewis 2005, Horton et al. 2007), and populations in which phallus lengths diverge significantly may exhibit substantial levels of pre-insemination isolation, possibly because of an absence of mechanical fit between male and female genitalia (Horton and Lewis 2005). Thus, our choice of the *A. antevolens* population to be examined in the current study maximized the possibility for successful insemination between *A. antevolens* and *A. musculus*.

Field-collected females and males from a given population were put into a ventilated tupperware cage containing small pear seedlings infested with eggs and nymphs of pear psylla, *Cacopsylla pyricola* (Förster) (Hemiptera: Psyllidae). After a 48 h interval, females were removed from the cage and placed individually in 135 ml ventilated cages each containing a pear seedling to act as an oviposition substrate. Seedlings were infested with immature psyllids. The egg-laden seedlings were then moved into a common rearing cage (containing eggs and offspring of all ovipositing females from that population) to obtain second-generation adults. Parental females were moved individually onto fresh seedlings to obtain additional eggs. Offspring were fed pear psylla supplemented with aphid-infested clippings from local *Salix*. The four populations were kept in separate environmental chambers throughout the rearing at 16:8 (L:D) h photoperiod and a temperature of 22–25°C. All studies except the mating trials and mtDNA study (see below) used first-generation (F1) males from the field-collected females. The mating trials, which required a large number of insects, used primarily F1 and F2 insects, with a small number of F3 bugs at the end of the trials used to reach desired final sample sizes. New generations were initiated by randomly selecting 10–20 females and males from the preceding generation to act as oviposition stock. Morphometric, genitalic, and genetic assessments (made using F1 males) had to include siblings in the samples, given the small sample sizes used to initiate the original cultures (especially the Michigan and Montana populations of *A. musculus*).

Pubescence.—To confirm our identifications of the two species, we measured length and density of setae on the endocorium, and density of setae on the cuneus (Fig. 1). We randomly chose 15 first-generation males from each culture for these measurements. These same males were then used for examination of genitalia and morphology. Methods for quantifying length and density of setae are provided in detail elsewhere (Horton et al. 2007). In each male, the right wing was detached from the insect and placed on a microscope slide. The wing was then photographed beneath a compound microscope at 100× (setal densities) or 200× (setal lengths). A copy of each photograph was printed, and setal characteristics examined on the photographs. Hair density on the endocorium was determined in a four-sided area (shaded trapezoid in Fig. 1). The area was defined by an imaginary line drawn through the cuneal break, a second line drawn 90 mm anterior of the first line and parallel to it, and two lines running anterior to posterior that connected the parallel lines. Density of setae on the cuneus was determined from the photographs for a 0.062 mm^2 circle approximately centered on the cuneus (Fig. 1). Length of setae on the endocorium was determined by measuring four setae located approximately at the center of the corium (Fig. 1). In both species, a distinctively long seta (hereafter, the "reference seta") occurs approximately half-way between the embolium and clavus, midway between the cuneal break and the point at which the epipleural fold crosses the medial furrow. We measured length of the four setae nearest this reference seta, and averaged the four lengths for each specimen.

Male genitalia.—In each of the 15 males per population, length of the phallus was estimated for the dissected organ using a dissecting microscope equipped with a micrometer. Dissection

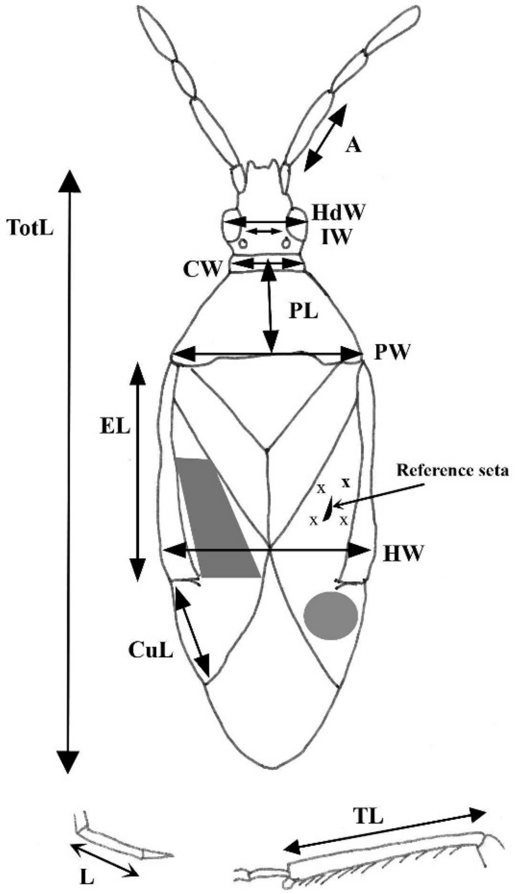

Fig. 1. Twelve mensural characters measured in male *A. musculus* and *A. antevolens*. Gray trapezoid and circle show locations on the hemelytra in which setal densities were estimated (all setal characteristics actually recorded for right forewing; trapezoid is shown on left forewing to reduce clutter). Setal length was estimated from the four setae (shown as x's) located near the long reference seta found roughly at midpoint of endocorium. TotL, total length; HdW, head width; IW, interocular width; CW, collar width; PW, pronotal width; PL, pronotal length; HW, hemelytral width; CuL, length of cuneus; EL, length of embolium; A, length of second antennal segment; L; length of penultimate segment of labium; TL, tibial length.

methods and illustrations of the phallus were given by Horton and Lewis (2005) and Horton et al. (2007). The phallus in these species is a membranous, two-walled organ that must be fully inflated within the female to allow insemination

(Horton and Lewis 2005). The dissected organ is measured as a single-walled structure (the form in which it is stored in the genital capsule); thus, lengths reported here for the dissected organ are approximately twice the length of the fully inflated organ.

The clasper is a sclerotized structure at the end of the male's abdomen, used to channel the phallus into the female's copulatory tube. Claspers vary extensively in appearance among species of *Anthocoris*, and are used to confirm identifications (Hill 1966, Kelton 1978). The clasper from each male was removed using microdissection tools and placed on a microscope slide (Horton and Lewis 2005). Claspers were then placed beneath a Leica DMLS compound microscope (Leica, Bannockburn, IL) and photographed with a Spot Insight color digital camera (Diagnostic Instruments, Sterling Heights, MI).

Body measurements.—For each of the 15 males per population used to assess pubescence characteristics and characteristics of the genitalia, we also measured 12 mensural characters (Fig. 1), as done by Horton et al. (2007). The specimens were mounted onto points. Measurements were made using a dissecting microscope equipped with a micrometer. Tibia length was measured on the rear left leg. Pronotal length included the collar because of the difficulty in objectively defining the break between the pronotum and collar in some specimens. Hemelytral width was measured at the point on the bug of maximum width. Length of the labium was recorded for the penultimate segment, whereas the antennal measurement was made for the second segment.

Mating trials.—All possible intra- and interpopulation crosses were made among the four populations, producing 16 possible crosses. We examined whether type of cross affected percentage of males attempting to mate and percentage of attempts leading to insemination. Matings were done using methods developed earlier (Horton et al. 2005). Virgin females and males of 2–5 d in age were used in the assays. We obtained unmated adults of the appropriate age by placing late instars from the cultures singly into petri dishes (9 cm diam). Psyllid-infested leaves were provided for food. As new adults emerged in the petri dishes, date of eclosion and sex of the insect were recorded.

Mating assays were done in plastic petri dishes (6 cm diam) at 22–24°C under fluorescent lighting. Females were placed singly into the petri dishes and allowed to settle. After 15 min, a male from the same population or from a different population was added. Each pair was allowed 30 min to initiate copulation. If the male failed to attempt copulation within 30 min, the assay was recorded as "no attempt." For males that attempted to mate, the female was dissected at the end of the assay to determine if the male had inseminated her (Horton and Lewis 2005). We previously used this measure of mating success (Horton and Lewis 2005, Horton et al. 2005) to show preinsemination reproductive incompatibility between populations of *A. antevolens*. Sample sizes were 22–25 pairings per type of cross.

Mitochondrial DNA.—Ten F1 males from each of the Maine, Michigan, Montana, and Washington cultures were used for mtDNA sequencing; the 10 males were randomly selected from the 15 males in each population used for examination of pubescence and morphology. An additional five specimens of *A. musculus* from Lolo, MT, and five specimens from Searsport, ME, that had been collected in 1999 (from collection of T.M.L.) were also analyzed, due to concerns about size of the field sample used to establish the cultures in the present study (see above: Source of insects and rearing). We had no extra specimens of Michigan bugs.

Sequence methods were described fully by Horton et al. (2007). Three legs from one side of each pinned specimen were used for the extractions (DNeasy kit, Qiagen Inc., Valencia CA). DNA sequences from these extracts were collected from the mitochondrial genes cytochrome oxidase subunit 1 (CO1) and cytochrome B (CytB). The primers C1-J-1751 [GGATCACCTGATA TAGCATTCCC] with C1-N-2191 [CCCGGTAAAATTAAAATATAAACTTC] were used to amplify ~450 bp of the CO1 gene; CB-J-10933 [TATGTACTACCATGAGGACAAATATC] with CB-N-11367 [ATTACACCTCCTAATTTATTAGGAAT] were used to amplify ~450 bp of CytB. Primer names correspond to those of Simon et al. (1994). Sequence alignments were made with ClustalW and edited visually using the program Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were trimmed to 423 bp for CO1 and 340 bp for CytB, retaining regions that showed high-quality sequence. Gene trees from the mtDNA sequence differences were created using neighbor-joining distance methods (NJ) implemented in MEGA3 (Kumar et al. 2004), Bayesian Monte-Carlo methods (BMC) using MrBayes3 (Ronquist and Huelsenbeck 2003), and maximum parsimony (MP) in PAUP4.10 (Swofford 2003). The data for each gene region were analyzed separately and combined by NJ; with MP and BMC, methods characters were analyzed as gene-specific partitions and combined. Site-specific differences among clades for the 763 bp were described using SITES software (Hey and Wakeley 1997).

Comparisons with additional populations of *A. antevolens*.—Morphological, mtDNA, and mating data collected for three Washington populations of *A. antevolens* were taken from Horton et al. (2005, 2007). Specimens were collected in 2002 (one year preceding collections made for the current study) from three populations, designated here and in the earlier publications as AL (Alder Lake), GC (Golf Course), and UG (Union Gap). The UG insects are from the same geographic location used in the current study as our source of *A. antevolens*. The UG and GC populations are sympatric in certain areas of the Yakima Valley (Yakima County, Washington), but can be separated by pubescence and characteristics of the male genitalia (Horton et al. 2007). The Alder Lake insects were collected 120 km west of the UG and GC populations. Complete descriptions of collecting sites for these three *A. antevolens* populations were provided by Horton et al. (2005, 2007).

Statistical methods.—Setal lengths, setal densities, phallus lengths, and body measures were compared between species and among populations using analysis of variance (ANOVA). For each trait, we extracted a single df contrast to compare *A. antevolens* to the mean of the three *A. musculus* populations. A second contrast was extracted to assess whether traits varied among the three *A. musculus* populations. A third contrast was used to compare the three *A. antevolens* populations that were examined by Horton et al. (2007). Total length was included as a covariate in tests on body measurements (ANCOVA).

A multivariate analysis of population and species differences in phallus lengths (1 trait), pubescence (3 traits), and body measurements (12 traits) was made using principal components analysis (PCA). The PCA was done using the correlation matrix. Components having eigenvalues greater than 1.0 were retained. Interpretation of components was assessed by examination of factor loadings after varimax (orthogonal) rotation. Mean factor scores were then compared among populations or between species using ANOVA and contrasts, as described above for the univariate analyses. The analyses were done using PROC FACTOR and PROC GLM in SAS (SAS Institute 2002).

Voucher specimens.—Voucher specimens from each population were deposited in the M.T. James Collection, Washington State University, Pullman. DNA sequence data for unique haplotypes were submitted to GenBank.

Results

Pubescence.—Length and density of setae on the endocorium differed between the Washington (*A. antevolens*) population and the three *A. musculus* populations (Fig. 2, Table 1). Setae were longer and at higher density in *A. antevolens* than *A. musculus* (Fig. 2, Table 1), confirming our identifications using Hill (1957) or Kelton (1978). Mean setal lengths also differed among the three *A. musculus* populations (Table 1), with setae slightly longer in bugs from Montana than in bugs from either Maine or Michigan (Fig. 2). Density of setae on the cuneus was significantly higher in the Union Gap (*A. antevolens*) population than the three *A. musculus* populations, whereas densities were similar among the three *A. musculus* populations (Fig. 2, Table 1). Photographs of the endocorium are shown for a male from the Washington *A. antevolens* population and a male from the Maine population of *A. musculus* to show the differences between the two species (Fig. 3). Length and density of setae on the endocorium also differed among the three *A. antevolens* populations that were examined in the earlier study (Fig. 2, Table 1).

Male genitalia.—Mean length of the dissected phallus differed among the three *A. musculus* populations (Fig. 4, Table 1), due to differences between the Montana insects and those from the Maine and Michigan sites (confirmed by single df contrasts at $P < 0.01$). Phallus length in *A. antevolens* (UG) was

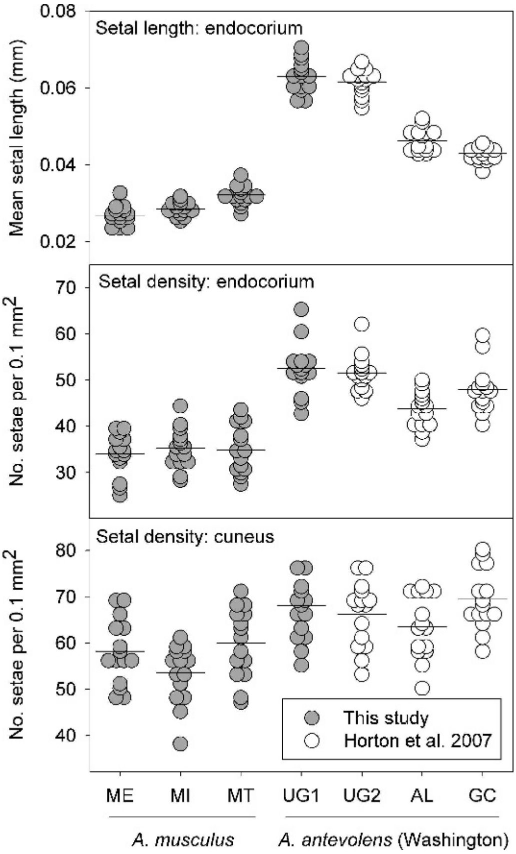

Fig. 2. Filled symbols: setal lengths and densities on endocorium, and setal densities on cuneus, with associated means (horizontal lines) for males of *A. antevolens* (Union Gap, Washington [UG1]) and *A. musculus* (Maine, Michigan, Montana). Open symbols: the same traits for males from three populations of *A. antevolens* (data from Horton et al. 2007).

similar to mean length for males from the three *A. musculus* populations (Table 1). Phallus lengths in Alder Lake and Golf Course *A. antevolens* were substantially longer than in males from the other populations.

Claspers were relatively similar in appearance among the three populations of *A. musculus* (Fig. 5), although claspers in males from Maine appeared to have been slightly more slender than those in *A. musculus* from Michigan and Montana. Males from the Washington (UG) population of *A. antevolens* had claspers

Table 1. *F*- and *P*-statistics from comparisons of setal measures, phallus lengths, and body measures between *A. antevolens* and *A. musculus*, among populations within *A. musculus*, and among populations within *A. antevolens*.

	This study				Horton et al. (2007)	
	A. antevolens (UG) vs 3 *A. musculus* populations		3 *A. musculus* populations		3 *A. antevolens* populations	
	$F_{1,98}$	P	$F_{2,98}$	P	$F_{2,98}$	P
SETAE						
Length (endocor.)	1616.7	<0.0001	13.8	<0.0001	186.6	<0.0001
Density (endocor.)	154.9	<0.0001	0.3	0.76	11.8	<0.0001
Density (cuneus)	26.1	<0.0001	3.2	0.05	2.6	0.08
PHALLUS LENGTH	2.1	0.15	6.4	0.003	99.4	<0.0001
BODY MEASURES[1]						
Total length	1.8	0.18	1.4	0.26	1.7	0.19
Collar width	14.3	0.0003	0.9	0.43	0.6	0.53
Hemelytral width	0.7	0.42	0.9	0.40	1.2	0.30
Head width	1.3	0.26	1.9	0.16	5.9	0.004
Pronotal width	3.6	0.06	0.9	0.41	0.3	0.73
Interocular width	0.4	0.51	0.1	0.97	2.0	0.15
Pronotal length	2.1	0.15	0.5	0.59	2.3	0.11
Cuneus length	7.6	0.007	1.1	0.34	21.7	<0.0001
Embolium length	3.2	0.08	1.5	0.23	4.3	0.016
Antenna	15.6	0.002	0.5	0.61	5.0	0.009
Tibial length	0.2	0.67	0.1	0.94	15.5	<0.0001
Labium	0.3	0.59	9.4	0.0002	2.6	0.08

[1] ANOVA used on total length; ANCOVA used on all other body measures, with total length as a covariate.

similar in size and shape to those in the *A. musculus* males. Males of *A. antevolens* from Alder Lake had substantially larger claspers than observed in other *A. antevolens* or in *A. musculus* (Fig. 5).

Body measurements.—There was substantial overlap among populations and between species in all measurements (Fig. 6). Despite this variation, ANOVA showed that traits often differed between species or among populations within species (Table 1). Within *A. musculus*, length of the labium differed significantly among populations (Table 1: $P = 0.0002$), associated with an increase in length from east to west populations (Fig. 6). Populations of *A. antevolens* differed significantly in several traits (Table 1; Fig. 6), including head width, and length of the cuneus, embolium, antennae, and tibia.

Multivariate summary of morphological and pubescence traits.—The first four components from the PCA displayed eigenvalues greater than 1.0 and accounted for 69% of the total variance in the 16 traits. Factor loadings following varimax rotation are shown for loadings exceeding 0.50 (Table 2; loadings multiplied by 100 for presentation). Two measures (embolium length and cuneus length) showed high loadings on more than one factor component (Table 2), and will be ignored in interpreting loadings. Collar width, pronotal width and length, hemelytral width, and tibial length were found to load on the first component, suggesting that this component described variation in body robustness and length of legs. The second component was associated with variation in pubescence. The third component described variation in total length, and length of labium and antennae. Finally, the fourth component captured variation

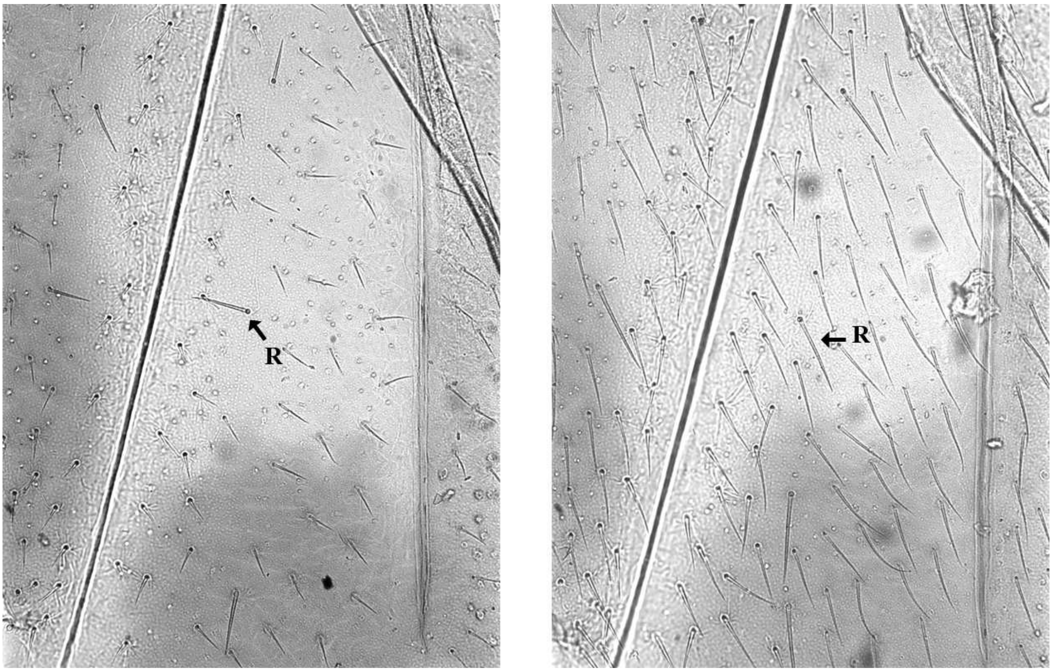

Fig. 3. Photograph of setae on hemelytra on a male *A. musculus* from Maine (left panel) and a male *A. antevolens* from Union Gap, Washington (right panel).

in phallus length and robustness of the head.

Mean scores for each component were then compared among populations and between species using ANOVA (Fig. 7, Table 3). The UG scores include those calculated using the Horton et al. (2007) results (= UG2 in Fig. 7) and those calculated from data collected in the current study (= UG1). The analyses showed that mean scores varied both between species and among populations within species (Table 3). While the 3 populations within *A. musculus* tended to cluster (Fig. 7), they also varied statistically in some traits, specifically in pubescence and in length of body and labium (Table 3): *A. musculus* from Montana had longer setae on the endocorium (Fig. 2), and had a longer body and labium (Fig. 6) than *A. musculus* from Maine and Michigan. Within *A. antevolens*, populations varied in pubescence (long and dense, especially in the Union Gap population), and in phallus length and robustness of the head. The two UG means in each component clustered together ($P > 0.20$

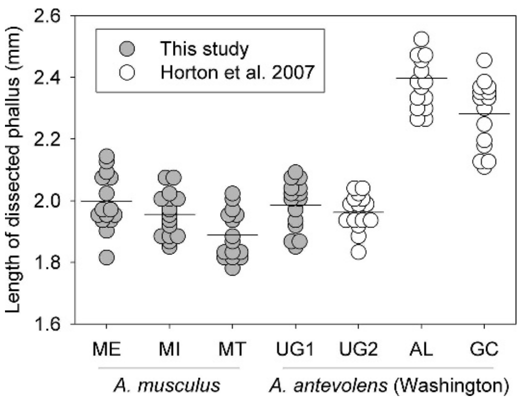

Fig. 4. Filled symbols: lengths of dissected phallus with associated means (horizontal lines) for males of *A. antevolens* (Union Gap, Washington [UG1]) and *A. musculus* (Maine, Michigan, Montana). Open symbols: phallus lengths in males from three populations of *A. antevolens* (data from Horton et al. 2007).

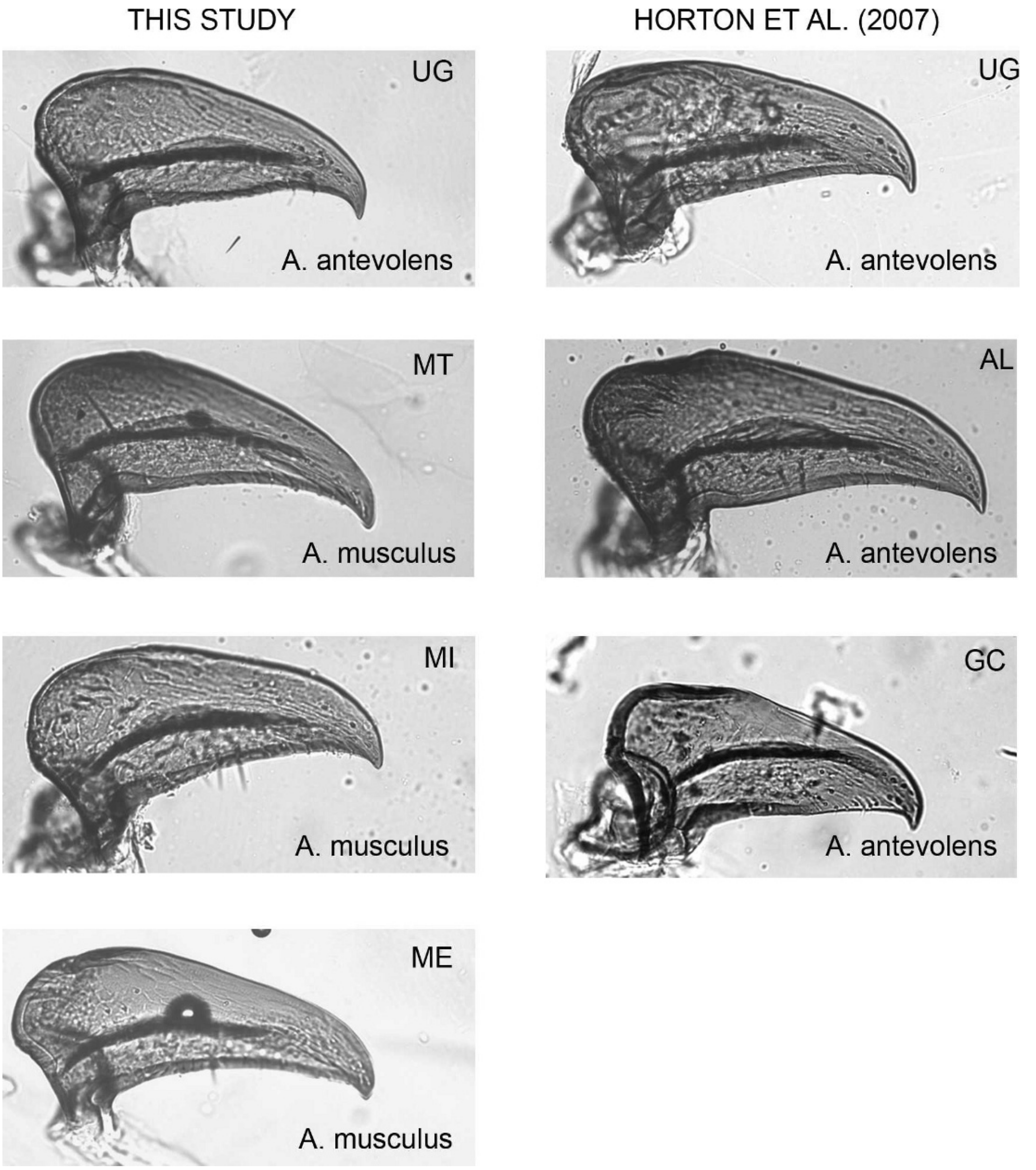

Fig. 5. Left column: photographs of claspers from representative males of *A. musculus* (Maine, Michigan, Montana) and a representative male of *A. antevolens* from Union Gap, Washington (UG). Right column: photographs of claspers from representative males of *A. antevolens* from three Washington populations (from specimens examined by Horton et al. 2007).

for all four components), suggesting that morphological and pubescence traits were similar in the two collections of Union Gap bugs.

Mating trials.—Males attempted to mate with females in at least 68% of pairings, irrespective of cross (Fig. 8A). Insemination success varied between 13% (in the *A. antevolens* [UG1] female x *A. musculus* [ME] male cross) to more than 90% in the Montana intrapopulation cross (Fig. 8A). The intrapopulation

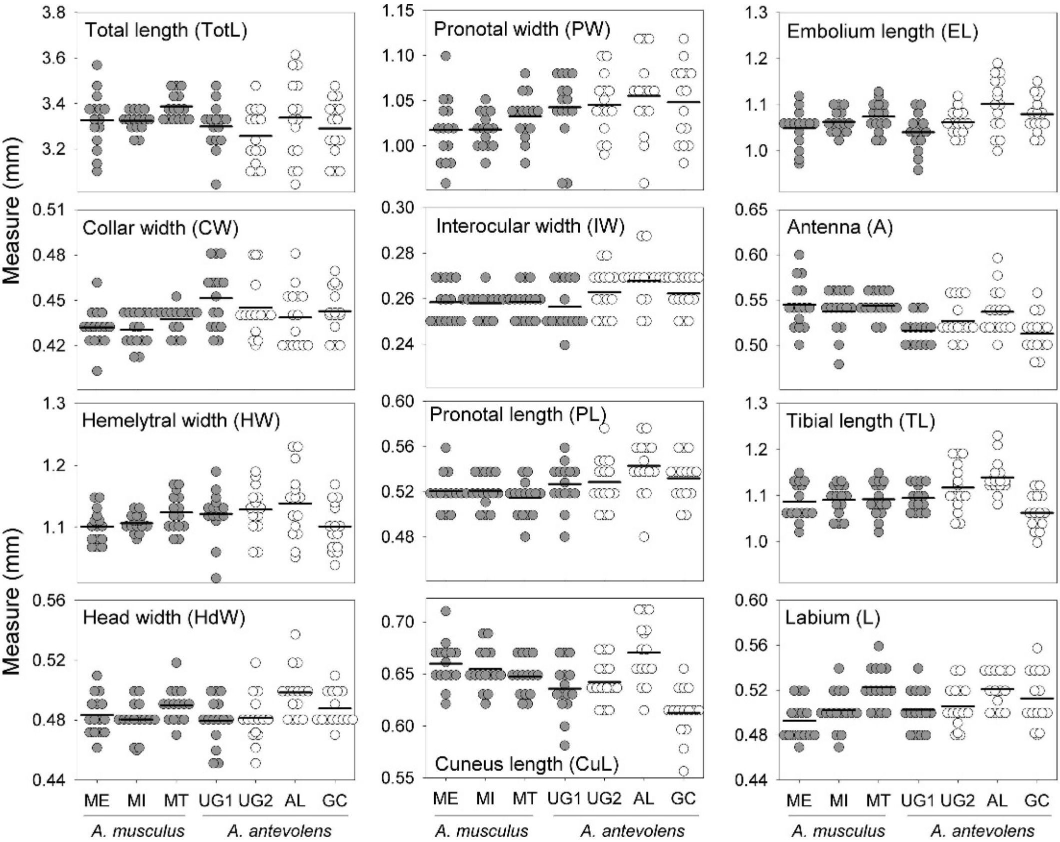

Fig. 6. Filled symbols: measurements for 12 mensural characters and associated means (horizontal lines) in males of *A. antevolens* (Union Gap, Washington [UG1]) and *A. musculus* (Maine, Michigan, Montana). Open symbols: the same traits for males from three populations of *A. antevolens* (data from Horton et al. 2007).

crosses tended to be highly successful, although some of the interpopulation crosses approached the success seen in the intrapopulation crosses. Pairings of *A. antevolens* with *A. musculus* led to insemination in at least some matings within each type of cross (Fig. 8A). Crosses between male *A. musculus* (MT, ME, MI) and female *A. antevolens* (UG1) resulted in insemination more often than crosses between male *A. antevolens* (UG1) and female *A. musculus* (MT, ME, MI). We observed high rates of insemination in all 9 crosses among the *A. musculus* populations (Fig. 8A). In the *A. antevolens* crosses from the Horton et al. (2005) study, interpopulation crosses involving the Union Gap (UG2) bugs were almost invariably unsuccessful, despite attempts at mating by most males (Fig. 8B).

Mitochondrial DNA.—Eight unique haplotypes from the combined CO1 and CytB sequences were obtained from the 32 individuals of *A. musculus* that were successfully sequenced for both gene regions. A summary gene tree derived using these eight haplotypes plus the seven haplotypes described for the UG, GC, and AL populations of *A. antevolens* (from sequence data in Horton et al. 2007) is shown in Fig. 9. The UG designation in Fig. 9 includes insects from the present study and insects

Table 2. Factor loadings (×100) from principal components analysis of 16 traits measured on *A. musculus* (3 populations) and *A. antevolens* (4 populations). Includes measurements for 3 *A. antevolens* populations reported elsewhere (Horton et al. 2007).

Measure	Factor 1	Factor 2	Factor 3	Factor 4
Phallus length	–	–	–	85
Setal length (endocor)	–	76	–	–
Setal density (endocor)	–	88	–	–
Setal density (cuneus)	–	80	–	–
Total length	–	–	74	–
Head width	–	–	–	51
Interocular width	–	–	–	64
Collar width	69	–	–	–
Pronotal width	71	–	–	–
Pronotal length	72	–	–	–
Hemelytral width	70	–	–	–
Embolium length[1]	–	–	*57*	*53*
Cuneus length[1]	*50*	*−59*	–	–
Antennal length	–	–	57	–
Tibial length	63	–	–	–
Length of labium	–	–	76	–
Variance (%)	20.7	18.0	16.9	13.3
Cumulative variance (%)	20.7	38.7	55.6	68.9

[1] Trait shows high loadings on more than one component.

collected in the year preceding the present study (from Horton et al. 2007). Positions of the 53 variable sites, as determined using SITES software, are shown in Table 4. The eight haplotypes from the *A. musculus* populations can be found in GenBank by accession number (CO1: EU598782-789; CytB: EU598790-797). Accession numbers for the *A. antevolens* haplotypes are available in Horton et al. (2007).

Structure of the gene tree was robust to the algorithm used in its estimation, as shown by comparing the boot-strap support for each clade (from the NJ analysis) and the clade credibility values (from the BMC analyses). The numbers of nucleotide substitutions are shown along each branch (Fig. 9). There were 23 and 22 parsimoniously informative substitutions in CytB and CO1, respectively. Similar trees were derived using other distance measures in NJ methods, in MP analyses, and when analyzed using partitioned and combined data in MP and BMC (not shown). All analyses showed clear support for a sister group relationship between the *A. musculus* populations (Maine, Michigan, Montana) and the Washington population of *A. antevolens* from Union Gap (UG labels in Fig. 9). The number of sequence differences between the *A. musculus* populations and the *A. antevolens* population from Union Gap (17–21 substitutions) is similar to that between two other Washington populations of *A. antevolens* (the GC and AL populations, with 18–23 substitutions). There were 24–34 substitutions between the GC-AL clade and the *A. musculus*-UG clade (Fig. 9). In contrast to these well-resolved clades, the branching orders and clustering within the three *A. musculus* populations depended upon the phylogenetic algorithm that was used, as is expected given the few differences seen among individual specimens.

Discussion

Questions about the taxonomic status of *A. musculus* and *A. antevolens* were

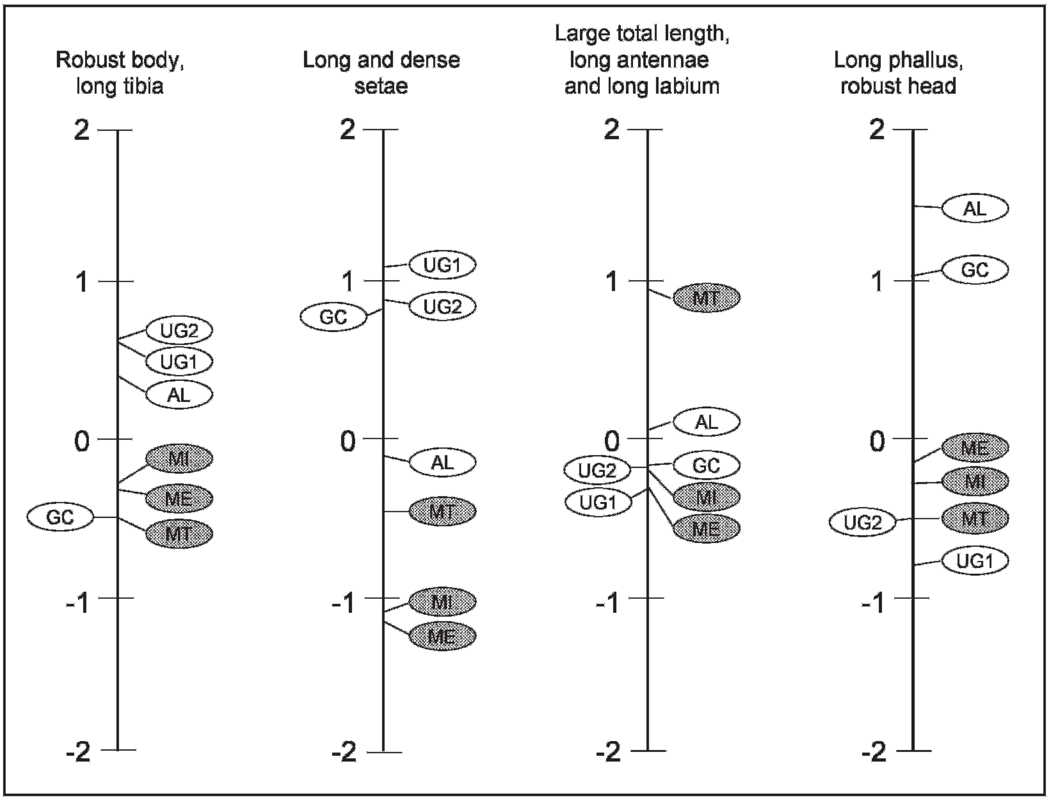

Fig. 7. Mean factor scores from principal components for three *A. musculus* populations (shaded ovals) and four *A. antevolens* populations (unshaded ovals); UG1 are scores for Union Gap insects using specimens collected in this study, while UG2 are scores calculated from traits measured on specimens collected by Horton et al. (2007). Statistical analyses of mean scores summarized in Table 3.

raised more than 40 years ago (Hill 1957, Anderson 1962). The present study is the first subsequent account to address this issue. The two species are separated in keys by characteristics of pubescence on the hemelytra (Hill 1957, Kelton 1978). *Anthocoris antevolens* has long and dense pubescence, whereas *A. musculus* has short and sparse pubescence (Hill 1957, Kelton 1978). Insects used in the present study readily separated into two pubescence classes (Figs. 2–3): *A. antevolens* (UG), with long and dense setae on the hemelytra; and *A. musculus* (MT, MI, ME), with short and sparse setae.

Despite the difference in pubescence between *A. musculus* and *A. antevolens*, there can be no doubt that *A. antevolens* and *A. musculus* are closely related (Hill 1957). The two species occur extensively on the same host taxa, especially members of the Salicaceae, Rosaceae, and Betulaceae (Anderson 1962, Kelton 1978, Horton et al. 2004). Both species are common inhabitants of pear orchards (Rasmy and MacPhee 1970, Horton and Lewis 2000). They both readily attack gall-forming aphids on poplar (Harper 1959, Alleyne and Morrison 1974, Horton and Lewis 2000), which appears to be a relatively uncommon behavior in North American species of *Anthocoris* (Horton and Lewis 2000, Horton et al. 2004). Size and shape of the male genitalia are similar in the two species, and differ from all North American *Anthocoris* species other than *Anthocoris dimorphicus* Anderson & Kelton

Table 3. *F*- and *P*-statistics from comparisons of factor scores between *A. antevolens* and *A. musculus*, among populations within *A. musculus*, and among populations within *A. antevolens*.

	This study				Horton et al. (2007)	
	A. antevolens (UG) vs 3 *A. musculus* populations		3 *A. musculus* populations		3 *A. antevolens* populations	
Factor	$F_{1,98}$	P	$F_{2,98}$	P	$F_{2,98}$	P
# 1 (body size, tibia)	13.7	0.0004	0.1	0.88	6.8	0.002
# 2 (pubescence)	162.5	<0.0001	6.8	0.002	19.0	<0.0001
# 3 (length, antenna, labium)	2.7	0.10	8.4	0.0004	0.3	0.77
# 4 (phallus, head size)	5.9	0.02	1.6	0.21	46.6	<0.0001

(Kelton 1978, T.M.L. unpublished data). Shape of the copulatory tube in females is similar between the two species, and differs from all other North American *Anthocoris* except *A. dimorphicus* (Ke and Bu 2007, T.M.L. unpublished data). Finally, males from one species readily inseminated females from the other species (Fig. 8A).

It is becoming increasingly clear, however, that many of these characteristics vary substantially. Length and density of setae on the hemelytra are highly variable geographically, which has led to confusion in assigning some populations to either species (Harper 1959, T.M.L. unpublished data). Pubescence also varies among populations within *A. musculus* or *A. antevolens* (Fig. 2; see also Horton et al. 2007). Size and shape of male genitalia vary geographically in this species group (Horton and Lewis 2005, Horton et al. 2007), as shown here especially for phallus length (Fig. 4). Kelton (1978) stated that the clasper in *A. musculus* is smaller than that of *A. antevolens*, but the accuracy of this statement appears to depend upon geographic source of the populations being compared (e.g., compare clasper size for *A. antevolens* [GC] and *A. musculus* males; Fig. 5).

We have shown complete preinsemination isolation among populations of insects that nonetheless all keyed to *A. antevolens* (Horton and Lewis 2005, Horton et al. 2005). Isolation extends even to populations that are sympatric (Horton and Lewis 2005, Horton et al. 2005), and occurs despite vigorous mating attempts made by males. In the

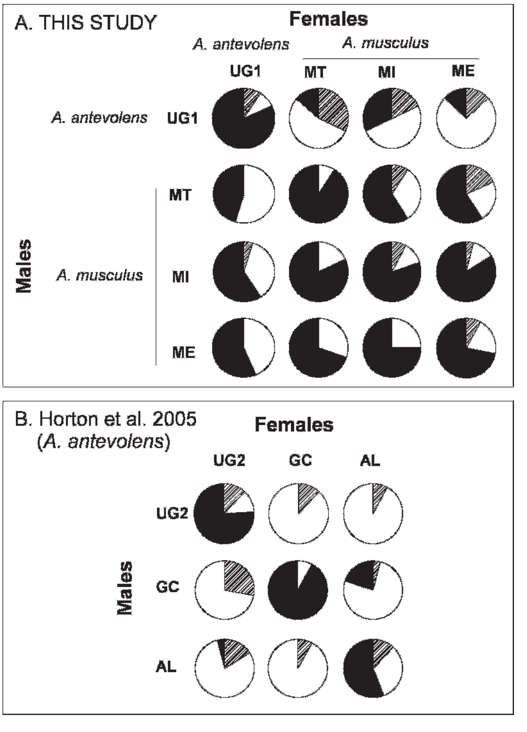

Fig. 8. (A) Percentage of crosses in which the male failed to attempt copulation (hatched area), attempted copulation but failed to inseminate the female (white fill), or successfully inseminated the female (black fill) for crosses involving *A. antevolens* (Union Gap, Washington [UG1]) and *A. musculus* (Maine, Michigan, Montana). Sample sizes were 22–25 pairings per type of cross. (B) Similar trials using three populations of *A. antevolens* (data in Horton et al. 2005).

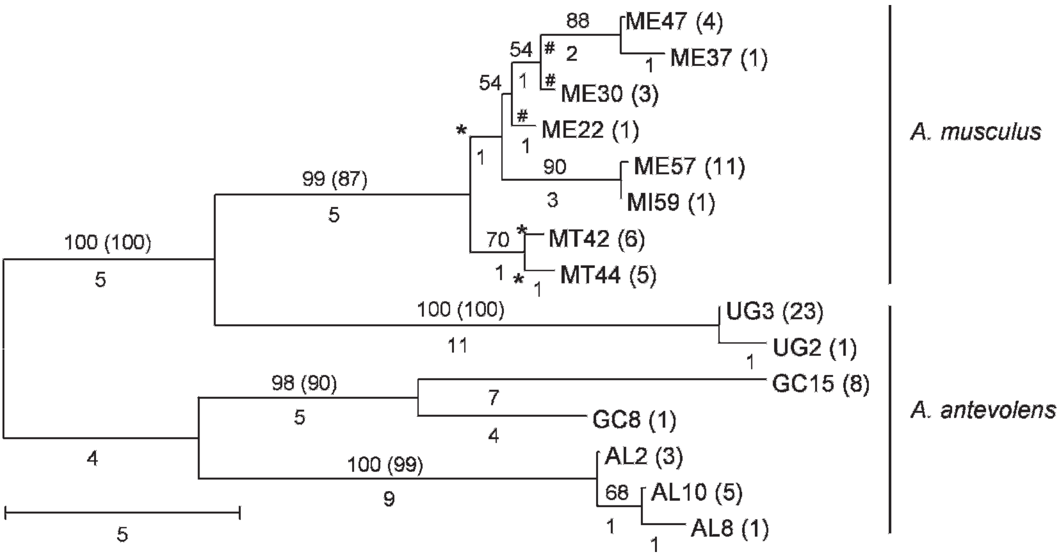

Fig. 9. mtDNA-based neighbor joining tree for specimens of *A. musculus* and *A. antevolens* collected in the present study and in Horton et al. (2007). Species designations based upon pubescence traits (Figs. 2–3; Hill 1957, Kelton 1978). Numbers following population acronyms are our specimen identifiers. Numbers in parentheses following a given specimen identifier indicate the total number of specimens having that particular haplotype; the ME57 haplotype includes 4 specimens from Maine and 7 specimens from Michigan. The tree was constructed using the number of nucleotide substitutions as the distance metric. Identical branch orders were obtained if the tree was developed using parsimony analysis (PAUP) or Bayesian analysis (MrBayes). Distances along the branches are shown below each branch. Bootstrap support from 1,000 runs is shown above the branch. Clade credibility from the Bayesian analysis is shown in parentheses above each branch. Asterisks depict an unresolved trichotomy from the Bayesian analysis; the # symbols indicate an unresolved trichotomy from the parsimony analysis.

present study, we actually obtained higher rates of insemination between *A. antevolens* (UG) and *A. musculus* than between *A. antevolens* (UG) and *A. antevolens* from other populations (Fig. 8A, B). This result appears to be consistent with our phallus data. Thus, successful insemination appears to be affected by whether populations have diverged significantly in phallus lengths (Horton and Lewis 2005, Horton et al. 2005). Divergence in phallus lengths may make it difficult for males to inseminate females from other populations, possibly due to physical mismatch of male and female genitalia (Horton and Lewis 2005). Similarities in phallus length between the three *A. musculus* and the Union Gap population of *A. antevolens* population may have been responsible in allowing insemination to occur in these crosses (Fig. 8A). It remains to be determined whether insemination in interpopulation crosses also leads to production of fertile offspring. Limited preliminary work suggests that crosses between *A. antevolens* (UG) and *A. musculus* from the three populations examined here fail to result in fertile eggs, even if the females are known to have been inseminated (T.M.L. unpublished data). The issue merits thorough examination.

The mtDNA data suggest that we have significant support for the existence of four distinct clades. Assuming that the mtDNA molecular clock for this group is 1–3% substitutions accumulated per million years (Gaunt and Miles 2002), then the *A. musculus* and *A. antevolens* clades nearest to one another (i.e., the UG and *A. musculus* clades) shared a common

Table 4. Itemized nucleotide substitutions among the 15 haplotypes represented in Fig. 9. Shown are replacements within the gene regions sequenced (1–423 for CO1, 424–763 for CytB): Replacement represents amino acid substitutions (R), synonymous (S) and unknown (?) due to unknown codon usage; informative = parsimony informative (Y = yes, N = no); Tstn/Tvrsn represent transversions (N) and transitions (v). Substitutions at positions 13–417 were in CO1 while the remaining substitutions were in CytB.

position	1111	1122222233	3333333344	4444445555	5555666666	677
position	1137893566	7745788900	4667788913	3456990015	6777001556	723
position	3815403928	5765359139	8365847375	9708594713	7036035456	898
Replacement	SSSSSSSSSS	??SSSS??SS	SSSSSSSSSS	SSSSSSSSRR	SSSSSSSSRS	===
Informative	YYYYYYYNNY	YYNNYYYYYN	YYYYNNYYYY	YYYYYYYYYN	YYYYYYYYYY	YYY
Tstn/Tvrsn	NNNNNvNvNN	NNNNNNNNNN	NNNNvNNNNN	NNNvNNNvNv	NNvvNNNNvN	NNN
Consensus	CGCAACCAAA	CATATACAGT	TCAGTATCAA	CAAATTGTAC	CCAAATAGAT	TCG
AL10	T-TGG-T---	T---C-T-A-	-TG----T-G	--GTCCAA--	-------AT-	CTA
AL2	T-TGG-T---	T---C-T-A-	-TG----T-G	--GTC-AA--	-------AT-	CTA
AL8	T-TGG-T---	T---C-T-A-	-TG----T-G	-GGTCCAA--	-------AT-	CTA
GC15	T-T--TTT--	---G-GT-AC	C--AGG---G	TG-TC-A---	-T--G--AT-	CTA
GC8	T-T--TTGG-	--C--GT-A-	C-G------G	T--TC-A---	----G--AT-	CTA
MT42	----G-----	----------	--------G-	----------	----------	---
MT44	----G-----	-------G--	--------G-	----------	----------	---
MI59	-A--------	-G-----G--	--------N-	----------	----------	---
ME47	-----A---G	-------G--	----------	-------A--	----------	---
ME22	-----A----	----------	----------	----------	----------	---
ME30	-----A----	-------G--	----------	----------	----------	---
ME37	-----A---G	-------G--	---A------	-------A--	----------	---
ME57	-A--------	-G-----G--	----------	----------	----------	---
UG2	T-T--TT---	----------	------C-G-	---C----GA	TTTT-CGA-C	--A
UG3	T-T--TT---	----------	------C-G-	---C----G-	TTTT-CGA-C	--A

ancestor 2–8 million years ago. The difference between the Golf Course and Alder Lake populations within *A. antevolens* is roughly of the same magnitude as that between the Union Gap population of *A. antevolens* and the *A. musculus* clade. Accumulated sequence differences should not automatically be used as an indicator of species status (Unruh and Woolley 1999, Cognato 2006). However, the sequence data in combination with morphological data (especially phallus length) and mating trials, provide strong support for the existence of multiple cryptic species within this complex.

In summary, we found relatively poor agreement between characteristics of pubescence (i.e., the trait used specifically in keys to separate *A. musculus* and *A. antevolens*; Hill 1957 and Kelton 1978) and other traits, including morphological characters, size and shape of the male genitalia, and mtDNA sequences. Differences in traits among populations within *A. antevolens* often exceeded differences in those traits between *A. antevolens* and *A. musculus*, if we continue to use pubescence to identify species. We conclude that much additional systematic work is required to adequately describe the taxonomy of bugs within this multi-species complex, and that available keys fail to adequately describe variation within this complex.

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